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(54) Title: STYRYL COMPOUNDS WHICH INHIBIT EGF RECEPTOR PROTEIN TYROSINE KINASE		
(57) Abstract <p>A method of inhibiting cell proliferation in a patient suffering from such disorder comprising administering to said patient an effective amount of a composition comprising, in admixture with a pharmaceutically acceptable carrier, a compound, or a pharmaceutically acceptable salt thereof, which is substituted styrene compound which can also be a naphthalene, an indane or a benzoxazine; including nitrile and molononitrile compounds, and pharmaceutical compositions comprising, in admixture with a pharmaceutically acceptable carrier, a pharmaceutically-effective amount of such compound.</p>		
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STYRYL COMPOUNDS WHICH INHIBIT
EGF RECEPTOR PROTEIN TYROSINE KINASE

FIELD OF THE INVENTION

This invention relates to the inhibition of cell proliferation. More specifically, this invention relates to the use of low molecular weight styryl compounds in inhibiting cell proliferation, including compounds which are
5 useful protein tyrosine kinase (PTK) inhibitors.

Normal cellular reproduction is believed to be triggered by the exposure of the cellular substrate to one or more growth factors, examples of which are insulin, epidermal growth factor (EGF) and platelet-derived growth factor
10 (PDGF). Such growth factors are typically specific for corresponding growth factor receptors which are imbedded in and which penetrate through the cellular membrane. The initiation of cellular reproduction is believed to occur when a growth factor binds to the corresponding receptor on the
15 external surface of the cellular membrane. This growth factor-receptor binding alters the chemical characteristics of that portion of the receptor which exists within the cell and which functions as an enzyme to catalyze phosphorylation of either an intracellular substrate or the receptor itself,
20 the latter being referred to as autophosphorylation. Examples of such phosphorylation enzymes include tyrosine

kinases, which catalyze phosphorylation of tyrosine amino acid residues of substrate proteins.

Many diseased states are characterized by the uncontrolled reproduction of cells. These diseased states involve a variety of cell types and include disorders such as leukemia, cancer, psoriasis, atherosclerosis and restenosis injuries. The inhibition of tyrosine kinase is believed to have utility in the control of uncontrolled cellular reproduction, i.e., cellular proliferative disorders.

Initiation of autophosphorylation, i.e., phosphorylation of the growth factor receptor itself, and of the phosphorylation of a host of intracellular substrates are some of the biochemical events which are involved in mitogenesis and cell proliferation. Autophosphorylation of the insulin receptor and phosphorylation of substrate proteins by other receptors are the earliest identifiable biochemical hormonal responses.

Elimination of the protein tyrosine kinase (PTK) activity of the insulin receptor and of the epidermal growth factor (EGF) receptor by site-directed mutagenesis of the cellular genetic material which is responsible for generation of insulin and EGF results in the complete elimination of the receptors' biological activity. This is not particularly desirable because insulin is needed by the body to perform other biological functions which are not related to cell proliferation. Accordingly, compounds which inhibit the PTK portion of the EGF receptor at concentrations less than the concentrations needed to inhibit the PTK portion of the insulin receptor could provide valuable agents for selective treatment of cell proliferation disorders.

Currently the chemotherapy of cancer makes use of inhibitors of DNA synthesis (e.g. adriamycin, fluorouracil) and compounds which disrupt the cytoskeleton (vinblastine). These compounds are highly toxic since their inhibitory activity is not limited to cancer cells, with the

distinction, however, that tumor cells are more readily attacked by the aforesaid inhibitors because these cells divide more rapidly and their DNA metabolism is consequently more active. A few types of cancers are treated with specific hormone derivatives. These cases, however, are the exception and the chemotherapeutic treatment for the majority of the various types of cancer is non-specific.

In the early 1980's it became apparent that 20 to 30 percent of cancers express characteristic oncogenic products which are growth factor receptors or their mutated homologs, and which exhibit PTK activity. The PTK activity is intrinsic to the receptor or its oncogene homolog and influences the cell proliferation via its PTK domain. Furthermore, each of these receptors (normal or mutated) exhibits a characteristic PTK activity with a distinct substrate specificity. One of these receptors is the epidermal growth factor (EGF) receptor and its oncogenic homolog V-ERB-B.

REPORTED DEVELOPMENTS

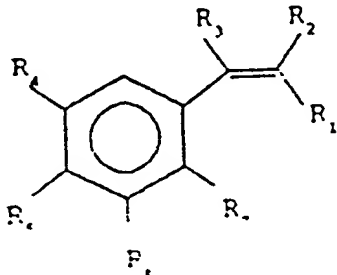
As a result of the above-described developments regarding the PTK activity of growth factor receptors, it has been proposed to treat cancer by means of various chemical substances capable of inhibiting the PTK activity of EGF. See, for example, Japanese patent Nos. 62-39523, 62-39558, 62-42923 and 62-42925. For example, aforementioned Japanese Laid-open Patent No. SHO 62-39558 discloses α -cyano-2,5-dihydroxycinnamamide as the active compound in compositions effective as PTK inhibitors.

The use of cinnamal malononitrile and various benzyliden malononitrile compounds as tumor growth inhibitors is disclosed in Gal et al., Studies on the Biological Action of Malononitriles. I. The Effect of Substituted Malononitriles on the Growth of Transplanted Tumors in Mice, *Cancer Research*, 12:565-72, 1952.

It has been reported that the most potent inhibitors of EGF receptors inhibit EGF-induced proliferation of A431/clone 15 cells with little or no effect on the proliferation of such cells when induced by other growth factors. It has been reported also that erbstatin inhibits the autophosphorylation of the EGF receptor in membranes of A431 cells. Low concentrations of erbstatin are required to inhibit EGF receptor autophosphorylation, whereas much higher concentrations of erbstatin are required to inhibit cyclic adenosine 10 3',5'-monophosphate (cAMP)-dependent protein kinase.

SUMMARY OF THE INVENTION

In accordance with the invention described and claimed in related abandoned application Serial No. 07/287,908, there is provided therein a pharmaceutical composition containing 15 as an active ingredient a compound of the general formula I



wherein:

R_1 and R_2 are each independently -CN, -CONH₂ or -COOH or on of

20 R_1 and R_2 may be -CSNH₂ or, when R_1 is -CN, R_2 can also be the group -C(NH₂)=C(CN)₂;

R_3 is -H, -CH₃ or -OH; and

R_4 , R_5 , R_6 and R_7 are each independently -H, -OH, C₁₋₅ alkyl, C₁₋₅

25 alkoxy, -NH₂, -CHO, halogen, -NO₂ or -COOH, or R_4 and R_5 together may represent a group -O-CH₂-O-;

provided that: (a) when R_4 and R_7 are each -OH, when R_3 , R_5 and R_6 are each -H and when one of R_1 and R_2 is -CN, then the other of R_1 and R_2 cannot be -CONH₂; and (b) when R_3 and R_7 are each -H, when R_5 is -OH and when R_4 and R_6 are both

5 -H or both C₁₋₅ alkyl, then R_1 is -CN and R_2 is -CN or the group -C(NH₂)=C(CN)₂; or

a pharmaceutically acceptable salt thereof.

Upon further analysis, there is provided, in accordance with the disclosure of the aforementioned parent application,
10 a novel pharmaceutical composition containing as an active ingredient a compound described by formula I above, or a pharmaceutically acceptable salt thereof, wherein:

R_1 and R_2 are each independently -CN, -CONH₂ or -COOH or one of

15 R_1 and R_2 may be -CSNH₂ or, when R_1 is -CN, R_2 can also be the group -C(NH₂)=C(CN)₂;

R_3 is -H, -CH₃ or -OH; and

R_4 , R_5 , R_6 and R_7 are each independently -H, -OH, C₁₋₅ alkyl, C₁₋₅

20 alkoxy, -NH₂, -CHO, halogen, -NO₂ or -COOH;

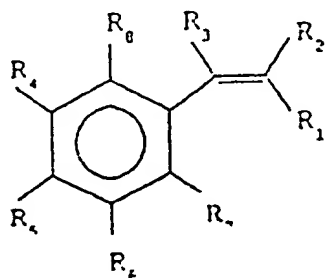
provided that: (a) when R_4 and R_7 are each -OH, when R_3 , R_5 and R_6 are each -H and when one of R_1 and R_2 is -CN, then the other of R_1 and R_2 cannot be -CONH₂; (b) when R_1 and R_2 are each -CN, then at least one of R_4 , R_5 , R_6 and R_7 is -NH₂,
25 -CHO, or -COOH; and (c) when R_3 and R_7 are each -H, when R_5 is -OH and when R_4 and R_6 are both C₁₋₅ alkyl, then R_1 is -CN and R_2 is -C(NH₂)=C(CN)₂.

There is also provided, in accordance with the
30 aforementioned parent application, a method of inhibiting cell proliferation in a patient suffering from such disorder

comprising administering to said patient a pharmaceutical composition as described above.

In accordance with the present invention, there is provided a method of inhibiting cell proliferation in a patient suffering from such disorder comprising administering to said patient an effective amount of a composition comprising, in admixture with a pharmaceutically acceptable carrier, a compound, or a pharmaceutically acceptable salt thereof, selected from the group consisting of

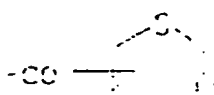
(A) a substituted styrene compound having the formula



II

one of R_1 and R_2 is alkyl, -H, -CN, -OH, -COOR, -CONRR or -CSNRR;

the other of R_1 and R_2 is alkyl, -H, -CN, -OH, -COOR, -CONRR, -CSNRR, -CHO, -CONHR, -CONHCH₂CN, -CH=C(CN)₂, -C(NH₂)=C(CN)₂, -NHCHO,



wherein R_1 and R_2 are not both alkyl, -H, -CN or -OH;

SUBSTITUTE SHEET

R is alkyl or -H;

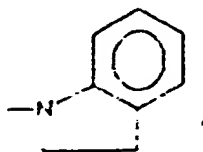
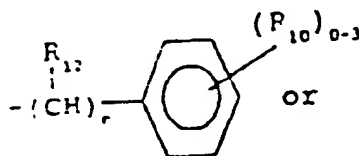
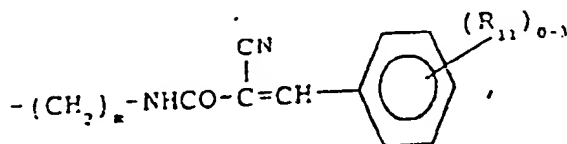
R₃ is alkyl, -H, -CN, -OH, -COOR, -CONRR, -CSNRR, -CH₂CN or -CH=C(CN)CONH₂;

5 R₄, R₅, R₆, R₇ and R₈ are each independently alkyl, -H, -CN, halo, -OR, -CHO, -COOH, -NRR, -NO₂, -NHCOCH₃, -SR, -CH=CHCOOH, -NHCO(CH₂)₂COOH or morpholino;

R₃ and R₇ together may be -CH₂CH₂-, -CH₂CH₂CH₂- or, starting from

R₃, -CONH-;

10 R₉ is -NH₂, -CONH₂,



R₁₀ is alkyl, halo, -OR, -COOR or -NO₂;

R₁₁ is alkyl, -CN, halo, -OR, -CHO, -COOH, -NRR, -NO₂, -NHCOCH₃, -SR, -CH=CHCOOH, -NHCO(CH₂)₂COOH or morpholino;

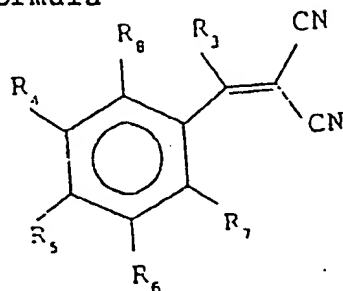
R₁₂ is alkyl, -H, halo, -OR or -COOR;

15 n is 0 to about 6; and

m is 2 to about 10;

provided that when each of R_4 and R_7 is $-OH$, when each of R_3 , R_5 , R_6 and R_8 is $-H$, and when one of R_1 and R_2 is $-CN$, then the other of R_1 and R_2 is not $-CONH_2$; and also provided that when one of R_1 and R_2 is $-CH=C(CN)_2$, at
 5 least one of R_3 , R_4 , R_5 , R_6 , R_7 and R_8 is not $-H$;

(B) an α -substituted benzylidene malononitrile compound having the formula



III

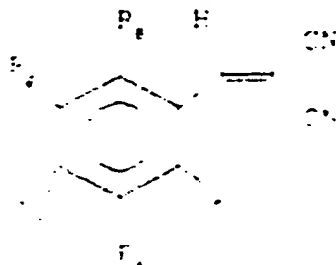
wherein:

R_3 is alkyl, $-CN$, $-OH$, $-COOR$, $-CONRR$, $-CSNRR$, $-CH_2CN$ or
 10 $-CH=C(CN)CONH_2$;

R is alkyl or $-H$; and

R_4 , R_5 , R_6 , R_7 and R_8 are each independently alkyl, $-H$, $-CN$,
 halo, $-OR$, $-CHO$, $-COOH$, $-NRR$, $-NO_2$, $-NHCOCH_3$, $-SR$,
 15 $-CH=CHCOOH$, $-NHCO(CH_2)_2COOH$ or morpholino;

(C) an α -unsubstituted benzylidene malononitrile compound having the formula



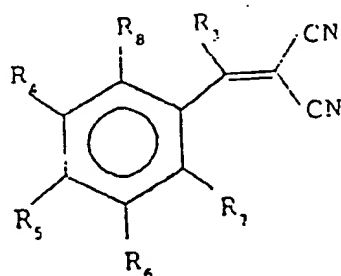
wherein:

at least one of R_4 , R_5 , R_6 , R_7 and R_8 is C_2 to about C_6 alkyl,
 20 $-CN$, $-CHO$, $-COOH$, $-NHR$, $-SR$, $-CH=CHCOOH$, $-NHCO(CH_2)_2COOH$
 or morpholino;

the remainder of R_4 , R_5 , R_6 , R_7 and R_8 are each independently alkyl, -H, -CN, halo, -OR, -CHO, -COOH, -NRR, -NO₂, -NHCOCH₃, -SR, -CH=CHCOOH, -NHCO(CH₂)₂COOH or morpholino; and

5 R is alkyl or -H; and

(D) a bicyclic benzylidene malononitrile compound having the formula



wherein:

10 R_3 and R_7 together are -CH₂CH₂-, -CH₂CH₂CH₂- or, starting from R_3 , -CONH-;

R_4 , R_5 , R_6 and R_8 are each independently alkyl, -H, -CN, halo, -OR, -CHO, -COOH, -NRR, -NO₂, -NHCOCH₃, -SR, -CH=CHCOOH, -NHCO(CH₂)₂COOH or morpholino; and

15 R is alkyl or -H.

It should be understood that formulae II, III, IV and V above encompass within their description compounds which are common to the description of the present application and the description of the aforementioned '908 parent application and
20 compounds which are first disclosed in the present application.

Another aspect of the present invention relates to pharmaceutical compositions comprising, in admixture with a pharmaceutically acceptable carrier, a pharmaceutically-
25 effective amount of a compound within the scope of formula II, III or V above, and within the scope of formula IV

provided that when R_5 is -OH and when one of R_4 or R_6 is halo, then the other of R_4 and R_6 is not tert-alkyl.

Compounds within the scope of the present invention have a specific affinity toward the substrate site of the tyrosine kinase domain of EGF receptors, inhibit EGF receptor kinase more than they inhibit PDGF receptor kinase and also effectively inhibit EGF-dependent autophosphorylation of the receptor.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphical representation of the activity of isolated EGFR kinases (given in percent of the total kinase activity) plotted against the concentrations in μM of 12 different inhibitors.

Figures 2a and 2b are graphical representations of the inhibitory effect of two pairs of tested compounds on the rate of the growth of KB and A431 cells respectively, the number of cells being plotted against time (in days).

Figure 3 is a graphical representation of the inhibition of A431 cell growth as a function of various concentrations (in μM) of the inhibitor "compound 2" according to the invention.

Figures 4a and 4b are graphical representations of the inhibitory effect of two pairs of tested compounds on the rate of the EGF-dependent proliferation of A431/clone 15 cells, with Figure 4a depicting inhibition effects of compounds found to inhibit EGF-dependent growth preferentially and Figure 4b depicting inhibition effects of compounds found to inhibit EGF-dependent growth exclusively.

DETAILED DESCRIPTION OF THE INVENTION

As employed above and throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

5 "Alkyl" means a saturated aliphatic hydrocarbon which may be either straight- or branch-chained containing from about 1 to about 6 carbon atoms.

"Lower alkyl" means an alkyl group as above, having 1 to about 4 carbon atoms which may be straight- or branch-chained
10 such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl or tert-butyl.

"Alkoxy" means an alkyl-oxy group in which "alkyl" is as previously described. Lower alkoxy groups are preferred. Exemplary groups include methoxy, ethoxy, n-propoxy, i-
15 propoxy and n-butoxy.

"Acyl" means an organic radical derived from an organic acid, a carboxylic acid, by the removal of its acid hydroxyl group. Preferred acyl groups are lower alkyl carboxylic acid groups such as acetyl and propionyl. Benzoyl is also
20 preferred.

"Halo" means a halogen. Preferred halogens include chloride, bromide and fluoride.

It is believed that therapeutically useful PTK inhibiting compounds should be competitive with the substrat
25 of EGF receptor tyrosine kinase (EGFRK) and not with adenosine triphosphate (ATP). The PTK inhibitors quercetin and genistein, which comp t with ATP, inhibit other protein kinases and as a result are highly cytotoxic. As a test of selectivity, compounds which inhibit EGFRK better than they
30 inhibit insulin receptor kinase (IRK) and/or PDGF receptor kinase are of considerable value.

It is theorized that solubility of the compounds of the present invention both in water and in mildly hydrophobic solvents will enhance the probability that they traverse the cell membrane. Various insoluble compounds, however, have
5 exhibited significant EGFRK inhibition in in vitro testing.

Disclosure of the Parent Application

Aforementioned application Serial No. 07/287,908 discloses, as a preferred class of compounds useful as the active ingredient in the pharmaceutical composition aspects
10 of that invention, those compounds described by formula I above in which at least one of R₁ and R₂ is -CN cis to the phenyl moiety of said formula, particularly those in which R₄ and R₅ are hydroxy groups, R₆ is hydrogen or hydroxy and R₃ and R₇ are hydrogens. Especially preferred pharmaceutical
15 compositions are disclosed therein as those containing as an active ingredient a compound, or a pharmaceutically acceptable salt thereof, selected from:

- α -hydroxy-3,4,5-trihydroxybenzylidene malononitrile;
- 3-methoxy-4,5-dihydroxybenzylidene malononitrile;
- 20 α -cyano-3,4-dihydroxycinnamthioamide;
- α -cyano-3,4-dihydroxy cinnamamide;
- 3,5-di-tert butyl-4-hydroxybenzylidene malononitrile;
- 4-formylbenzylidene malononitrile;
- 3,4-methylenedioxy-6-nitrobenzylidene malononitrile;
- 25 3,4-dihydroxybenzylidene malononitrile;
- χ -cyano-8-amino-3,4-dihydroxycinnamal malononitrile;
- χ -cyano-8-amino-3,4,5-trihydroxycinnamal malononitrile;
- δ -cyano-8-amino-3,4-dihydroxy-5-methoxycinnamal malononitrile;
- 30 χ -cyano-8-amino-3,4-dihydroxy-5-bromocinnamal malononitrile;
- and
- χ -cyano-8-amino-3-hydroxy-4-nitrocinnamal malononitrile;

In accordance with another aspect of the invention disclosed and claimed in the aforementioned parent
35 application, there are also provided compounds of formula I

above, and pharmaceutically acceptable salts thereof, selected from:

- α -hydroxy-3,4,5-trihydroxybenzylidene malononitrile;
 α -cyano-3,4-dihydroxycinnamthioamide;
5 4-formylbenzylidene malononitrile;
3,4-methylenedioxy-6-nitrobenzylidene malononitrile;
 χ -cyano- β -amino-3,4-dihydroxycinnamal malononitrile;
 χ -cyano- β -amino-3,4,5-trihydroxycinnamal malononitrile;
 χ -cyano- β -amino-3,4-dihydroxy-5-methoxycinnamal
10 malononitrile; χ -cyano- β -amino-3,4-dihydroxy-5-bromocinnamal
malononitrile;
and
 χ -cyano- β -amino-3-hydroxy-4-nitrocinnamal malononitrile.

Preferred Compounds Useful in Practicing the Present

15 Invention

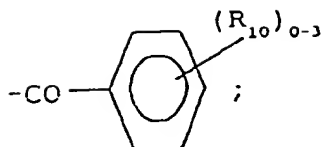
A preferred class of compounds useful in the practice of the present invention includes those compounds comprising (A) one or more cyano groups attached to the ethylene portion of the styryl compound, for example, styryl and cinnamylidene
20 nitriles and benzylidene and cinnamal malononitriles; and/or (B) one or more substituents on the phenyl portion of the styryl compound, for example, alkyl, cyano, halo, hydroxy, alkoxy, formyl, amino, alkylamino and nitro.

A preferred class of compounds useful in the practice of
25 the present invention can also be described as including compounds selected from the group consisting of

(A) those described by Formula II above where:

R_1 is -CN, -COOR, -CONRR or -CSNRR;

R_2 is -H, -CN, -COOR, -CONRR, -CSNRR, -CONHR, or



wherein R_1 and R_2 are not both $-\text{CN}$;

R is alkyl or $-\text{H}$;

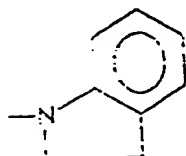
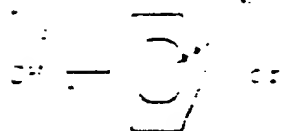
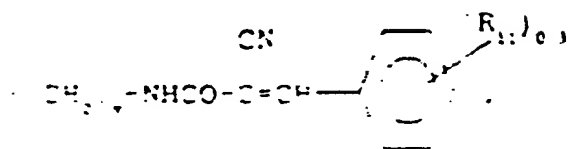
R_3 is $-\text{H}$;

R_4 , R_5 , R_6 , R_7 and R_8 are each independently alkyl, $-\text{H}$, halo
5 or $-\text{OR}$;

R_3 and R_7 together may be $-\text{CH}_2\text{CH}_2-$, $-\text{CH}_2\text{CH}_2\text{CH}_2-$ or, starting from

R_3 , $-\text{CONH}-$;

R_9 is $-\text{NH}_2$, $-\text{CONH}_2$,



10 R_{10} is alkyl, halo, $-\text{OR}$, $-\text{COOR}$ or $-\text{NO}_2$;

15

R_{11} is alkyl, halo or -OR;

R_{12} is alkyl or -H;

n is 0 to about 4; and

m is 2 to about 10;

5 (B) those described by formula IV above where:

at least one of R_4 , R_5 , R_6 , R_7 and R_8 is C_2 to C_6 alkyl;

the remainder of R_4 , R_5 , R_6 , R_7 and R_8 are each independently alkyl, -H, halo or -OR; and

R is alkyl or -H; and

10 (C) those described by formula V above where:

R_3 and R_7 together are $-CH_2CH_2-$, $-CH_2CH_2CH_2-$ or, starting from R_3 , $-CONH-$;

R_4 , R_5 , R_6 and R_8 are each independently alkyl, -H, halo or -OR; and

15 R is alkyl or -H.

Preferred compounds described by Formula III above include

(A) those compounds where:

20 R_3 is -CN, -OH, -COOR, -CONRR, -CSNRR, $-CH_2CN$ or $-CH=C(CN)CONH_2$; and

(B) those compounds where:

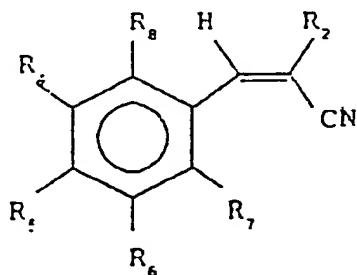
R_3 is alkyl;

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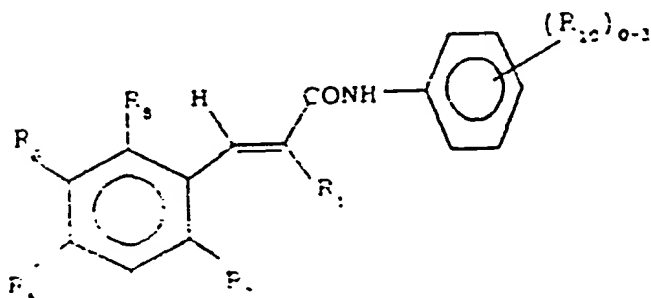
at least one of R_4 , R_5 , R_6 , R_7 and R_8 is C_2 to about C_6 alkyl, $-CN$, $-CHO$, $-COOH$, $-NHR$, $-SR$, $-CH=CHCOOH$, $-NHCO(CH_2)_2COOH$ or morpholino; and

5 the remainder of R_4 , R_5 , R_6 , R_7 and R_8 are each independently alkyl, $-H$, $-CN$, halo, $-OR$, $-CHO$, $-COOH$, $-NRR$, $-NO_2$, $-NHCOCH_3$, $-SR$, $-CH=CHCOOH$, $-NHCO(CH_2)_2COOH$ or morpholino.

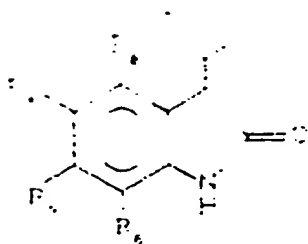
More preferred compounds of this invention include those of Formulae VI to X below:



VI

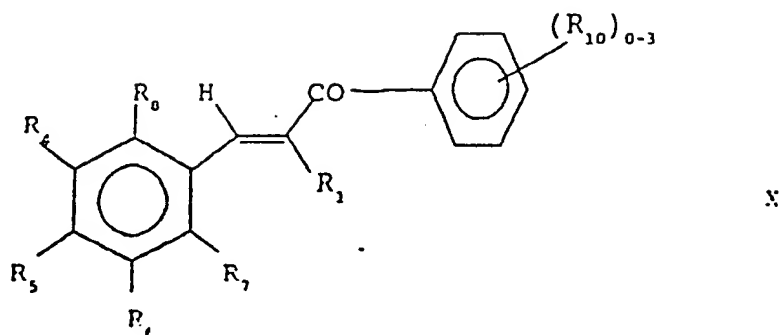
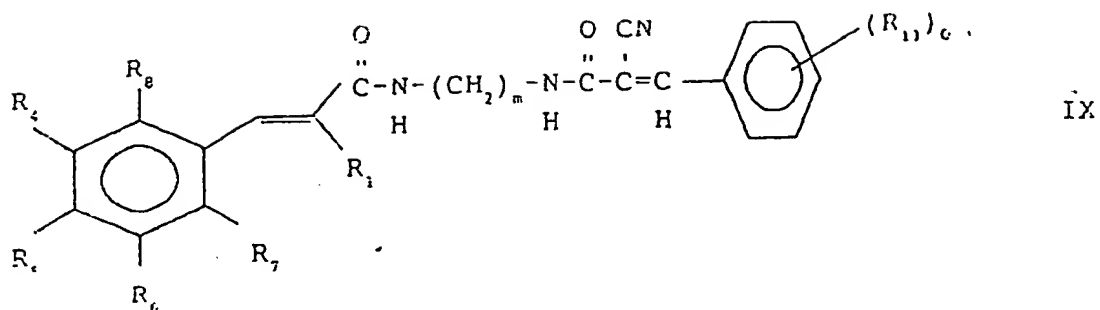


VII



VIII

SUBSTITUTE SHEET

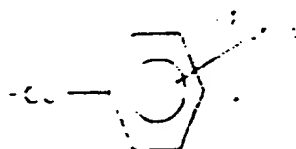


where R_1 , R_2 , R_4 , R_5 , R_6 , R_7 , R_8 , R_{10} , R_{11} and m are as described immediately above.

Even more preferred compounds are described by Formulae VI, VII, VIII and IX where:

5 R_1 is -CN, -COOR, -CONRR or -CSNRR;

R_2 is -H, -CN, -COOR, -CONRR, -CSNRR, -CONHR, or



wherein R_1 and R_2 are not both -CN;

R is alkyl or -H;

R₄, R₅, R₆, R₇ and R₈ are each independently lower alkyl, -H, -OH, lower alkoxy or halo;

R₁₀ is lower alkyl or -COOR;

5 R₁₁ is lower alkyl, -OH, lower alkoxy or halo; and

m is 2 to about 6.

The most preferred compounds are described by Formula VII where R₁ is -CN; R₄ and R₅ are each independently -OH; R₆, R₇ and R₈ are each independently -H; and there are no R₁₀ substituents, and Formula X where R₁ is -CN; R₄ and R₅ are each independently -OH; R₆, R₇ and R₈ are each independently -H; and there are no R₁₀ substituents.

Compounds of this invention may be useful in the form of the free base, in the form of a salt and as a hydrate. All forms are within the scope of the invention. Acid addition salts may be formed and are simply a more convenient form for use; in practice, use of the salt form inherently amounts to use of the base form. The acids which can be used to prepare the acid addition salts include preferably those which produce, when combined with the free base, pharmaceutically acceptable salts, that is, salts whose anions are non-toxic to the animal organism in pharmaceutical doses of the salts, so that the beneficial properties inherent in the free base are not vitiated by side effects ascribable to the anions. Although pharmaceutically acceptable salts of said basic compound are preferred, all acid addition salts are useful as sources of the free base form even if the particular salt per se is desired only as an intermediate product as, for example, when the salt is formed only for purposes of purification and identification, or when it is used as an intermediate in preparing a pharmaceutically acceptable salt by ion exchange procedures.

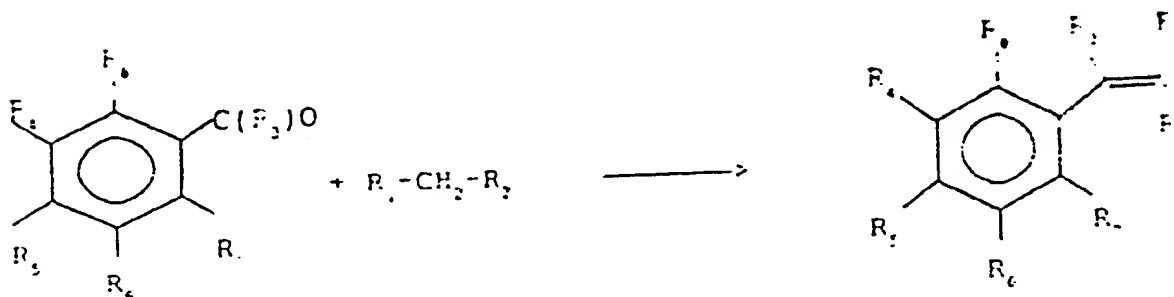
Pharmaceutically acceptable salts within the scope of the invention include those derived from the following acids: mineral acids such as hydrochloric acid, sulfuric acid, phosphoric acid and sulfamic acid; and organic acids such as
 5 acetic acid, citric acid, lactic acid, tartaric acid, malonic acid, methanesulfonic acid, ethanesulfonic acid, benzenesulfonic acid, p-toluenesulfonic acid, cyclohexylsulfamic acid, quinic acid and the like.

The corresponding acid addition salts comprise the
 10 following: hydrochloride, sulfate, phosphate, sulfamate, acetate, citrate, lactate, tartarate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, cyclohexylsulfamate and quinate, respectively.

The acid addition salts of the compounds of this
 15 invention are prepared either by dissolving the free base in an aqueous or aqueous-alcohol solution or other suitable solvents containing the appropriate acid and by isolating the salt by evaporating the solution or by reacting the free base and acid in an organic solvent, in which case the salt
 20 separates directly or can be obtained by concentration of the solution.

Compounds useful in the practice of this invention can be prepared by known methods, for example, Knoevenagel condensation reactions such as those disclosed in U.S. Patent
 25 No. 3,149,148.

Compounds of this invention may be prepared by the following reaction sequence:



Knoevenagel condensation of a substituted benzaldehyde in a polar media with an active methylene compound of the formula $R_1CH_2R_2$ in the presence of ammonia or amines such as piperidine and raised heat results in the products of this invention. When substitution of the R_3 group is desired, the corresponding ketone starting material is used. Reaction temperatures in the range of 25°C to reflux and reaction times vary depending on the materials being used in the condensation.

For example, the malonic acid derivatives of formula II above, can be prepared by reacting a corresponding substituted benzaldehyde with malononitrile to obtain the benzylidene derivatives or with malononitrile dimers to obtain the cinnamal derivatives. The reaction is generally carried out in a suitable solvent, such as ethanol or benzene, and in the presence of a catalyst, e.g., piperidine, pyridine or β -alanine. Alternatively, a suitably substituted benzoyl chloride can be reacted with malononitrile in the presence of an amine in a non-polar organic solvent.

Compounds of this invention are either commercially available, known in the literature or can be made by known procedures.

Various R , R_1 , R_3 , R_4 , R_5 , R_6 , R_7 , R_8 and R_{11} substituents on the phenyl ring or chain can be present in the starting compound or added after formation of the condensation product by methods known in the art for substitution or conversion of one group to another. If the substituents themselves are reactive, then the substituents can themselves be protected according to the techniques known in the art. A variety of protecting groups known in the art may be employed. Examples of many of these possible groups may be found in "Protective Groups in Organic Synthesis" by T. W. Green, John Wiley and Sons, 1981. For example, nitro groups can be added to the aromatic ring by nitration and the nitro group converted to other groups, such as amino by reduction, and halo by diazotization of the amino group and replacement of the diazo

group. Acyl groups can be substituted onto the aryl groups by Friedel-Crafts acylation. The acyl groups can then be transformed to the corresponding alkyl groups by various methods, including the Wolff-Kishner reduction and Clemmenson
5 reduction. Amino groups can be alkylated to form mono- and di-alkylamino groups and mercapto and hydroxy groups can be alkylated to form corresponding ethers. Primary alcohols can be oxidized by oxidizing agents known in the art to form carboxylic acids or aldehydes and secondary alcohols can be
10 oxidized to form ketones. Thus, substitution or alteration reactions can be employed to provide a variety of substituents throughout the molecule of the starting material, intermediates or the final product.

Compounds within the scope of this invention exhibit
15 significant activity as protein tyrosine kinase inhibitors and possess therapeutic value as cellular antiproliferative agents for the treatment of certain conditions including, for example, psoriasis and restenosis injuries. It is expected that the invention will be particularly applicable to the
20 treatment of atherosclerosis. With regard to the treatment of some conditions, for example, atherosclerosis, certain people may be identified as being at high risk, for example, due to genetic, environmental or historical factors. Compounds within the scope of the present invention can be
25 used in preventing or delaying the occurrence or reoccurrence of such conditions or otherwise treating the condition.

Compounds of the present invention can be administered to a mammalian host in a variety of forms adapted to the chosen route of administration, e.g., orally or parenterally.
30 Parenteral administration in this respect includes administration by the following routes: intravenous, intramuscular, subcutaneous, intraocular, intrasynovial, transepithelial including transdermal, ophthalmic, sublingual and buccal; topically including ophthalmic, dermal, ocular,
35 rectal and nasal inhalation via insufflation and aerosol and rectal systemic.

The active compound may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsules, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipient and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 6% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 1 and 1000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the following: a binder such as gum tragacanth, acacia, corn starch or gelatin; an excipient such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; a sweetening agent such as sucrose, lactose or saccharin; or a flavoring agent such as peppermint, oil of wintergreen or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active

compound may be incorporated into sustained-release preparations and formulations.

The active compound may also be administered parenterally or intraperitoneally. Solutions of the active
5 compound as a free base or a pharmacologically acceptable salt can be prepared in water suitably mixed with a surfactant such as hydroxypropylcellulose. A dispersion can also be prepared in glycerol, liquid polyethylene glycols and mixtures thereof and in oils. Under ordinary conditions of
10 storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile
15 injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and
20 fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the
25 use of a coating such as lecithin, by the maintenance of the required particle size in the case of a dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol,
30 phenol, sorbic acid, thimerosal and the like. In many cases it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by use of agents delaying absorption, for example, aluminum
35 monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered
5 sterilization. Generally, dispersions are prepared by incorporating the sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile
10 injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying technique which yield a powder of the active ingredient plus any additional desired ingredient from the previously sterile-filtered solution thereof.

15 The therapeutic compounds of this invention may be administered to a mammal alone or in combination with pharmaceutically acceptable carriers. As noted above, the relative proportions active ingredient and carrier is determined by the solubility and chemical nature of the
20 compound, chosen route of administration and standard pharmaceutical practice.

The dosage of the present therapeutic agents which will be most suitable for prophylaxis or treatment will vary with the form of administration, the particular compound chosen
25 and the physiological characteristics of the particular patient under treatment. Generally, small dosages will be used initially and, if necessary, will be increased by small increments until the optimum effect under the circumstances is reached. The therapeutic human dosage, based on
30 physiological studies using rats, will generally be from about 0.01 mg to about 100 mg/kg of body weight per day or from about 0.4 mg to about 10 g or and higher although it may be administered in several different dosage units from once to several times a day. Oral administration requires higher
35 dosages.

EXAMPLES

Embodiments of the present invention are described in the following non-limiting examples which include a description of pharmacological test procedures believed to correlate to therapeutic activity in humans and other animals.

Examples 1-5 and 15 illustrate compounds which are substituted benzylidene malononitriles, that is, R_1 and R_2 are each $-CN$. Examples 7-9, 13 and 14 illustrate compounds which are substituted 8-amino cinnamal malononitriles, that is, R_1 is $-CN$ and R_2 is $-C(NH_2)=C(CN)_2$. Examples 6, 10, 11, 16, 18 and 24 illustrate compounds wherein R_1 is $-CN$ and R_2 is $-CONH_2$, $-COOH$, $-CSNH_2$, $-CONHNH_2$, $-CONHCONH_2$ and $-CO-(\text{substituted phenyl})$, respectively. Example 12-C is a comparative compound and illustrates a benzylidene malononitrile wherein R_1 and R_2 together are $-O-CH_2-O-$. Example 17 illustrates a compound wherein R_1 is $-H$ and R_2 is $-CH=C(CN)_2$. Examples 19 and 22 illustrate indanes, that is, R_3 and R_7 together are $-CH_2CH_2-$. Examples 20 and 21 illustrate compounds wherein R_2 is $-COOR$. Example 23 illustrates a tetrahydronaphthalene, that is, R_3 and R_7 together are $-CH_2CH_2CH_2-$.

Example 13,4-Dihydroxybenzylidene malononitrile

To 11 g (80 mmol) of 3,4-dihydroxybenzaldehyde and 5.5 g (83 mmol) of malononitrile in 40 ml of ethanol, 7 drops of piperidine were added and the mixture was heated at $70^\circ C$ for 0.5-1 hour and then poured into water. The resulting solid precipitate was separated by filtration to give 12.7 g (86% yield) of a yellow solid, m.p. $225^\circ C$.

Examples 2-4

Following the same procedure as set forth for Example 1 above, the following were prepared: Example 2, 3-methoxy-4,5-dihydroxybenzylidene malononitrile, m.p. $235^\circ C$; Example 3, 3,5-di-tert butyl-4-hydroxybenzylidene malononitrile, m.p.

135°C; and Example 4, 3,5-di-tert butylbenzylidene malononitrile, m.p. 95-98°C.

Example 5

α -Hydroxy-3,4,5-trihydroxybenzylidene malononitrile

5 To 2 g (30 mmol) of malononitrile and 4 ml (40 mmol) of triethylamine in 100 ml of CH_2Cl_2 , triacetyl galloyl chloride (prepared from 7 g (24 mmol) of triacetyl gallic acid and thionyl chloride) in 50 ml CH_2Cl_2 was added. The resulting mixture was then stirred for two hours at room temperature,
10 poured into 50 ml water and hydrolyzed by heating for 2 minutes at 80°C with a solution of 2.5 g NaOH in 30 ml ethanol. The mixture was extracted with ethyl acetate and the organic extract was further worked up by washing with water, drying, filtering and evaporation. Chromatography on
15 silica gel gave 1.5 g (29% yield) of the product as an oily solid.

Example 6

α -Cyano-3,4-dihydroxycinnamamide

Reaction of 2.4 g (10 mmol) 3,4-dihydroxybenzaldehyde
20 and 0.9 g (10.7 mmol) cyanoacetamide by the procedure described in Example 1 above gave 1.45 g (70% yield) of product as a yellow solid, m.p. 247°C.

Example 7

χ -Cyano-8-amino-3,4-dihydroxycinnamal malononitrile

25 1.4 g (10 mmol) 3,4-Dihydroxybenzaldehyde, 1.4 g (10.6 mmol) malononitrile dimer and 0.3 g of 8-alanine in 50 ml ethanol were heated at 70°C for 40 minutes. 100 ml of water was added, the suspension cooled and a solid precipitate was filtered off, washed with water and dried to give 1.3 g (53%
30 yield) of a yellow-orange solid, m.p. 235°C.

Examples 8 and 9

Following the same procedure as described for Example 7 above, the following were prepared: Example 8, χ -cyano-8-amino-3,4,5-trihydroxycinnamal malononitrile, m.p. 275°C; and

Example 9, δ -cyano-8-amino-3-hydroxy-4-nitrocinnamal malononitrile, m.p. 219°C.

Example 10

α -Cyano-3,4-dihydroxycinnamic acid

5 (a) 2 g (15 mmol) 3,4-Dihydroxybenzaldehyde, 3 g (21 mmol) of tert-butyl cyanoacetate and 0.5 ml of piperidine in 50 ml ethanol were heated to reflux for 1 hour, poured into water, filtered, washed and dried to yield 2.5 g (yield 66%) of tert-butyl α -cyano-3,4-dihydroxycinnamate as a yellow
10 solid.

(b) 1.6 g of the tert-butyl ester from (a) in 10 ml of trifluoroacetic acid was stirred at room temperature for 20 minutes, 50 ml of H₂O was added and the cooled suspension was filtered, washed with water and dried to give 1 g (yield 85%)
15 of product as a yellow solid, m.p. 240°C.

Example 11

α -Cyano-3,4-dihydroxycinnamthioamide

To 0.83 g (6mmol) 3,4-dihydroxybenzaldehyde and 0.7 g (7 mmol) cyanothioacetamide in 30 ml ethanol was added 4 drops
20 of piperidine. The mixture was refluxed for 1 hour, poured into ice water, filtered and dried, giving 0.54 g (41% yield) of an orange solid, m.p. 213°C. Calculated analysis for C₁₀H₈N₂O₂S: C=54.54, H=3.64, N=12.73; found: C=54.44, H=3.87, N=12.91.

25

Example 12-C

3,4-Methylenedioxy-6-nitrobenzylidene malononitrile

1 g (5.1 mmol) 3,4-Methylenedioxy-6-nitrobenzaldehyde. 0.4 g (6 mmol) malononitrile and 0.2 g 8-alanine in 30 ml ethanol were stirred 16 hours at room temperature, 50 ml H₂O
30 was added and the reaction mixture was filtered, giving 1 g (80% yield) of a bright yellow solid, m.p. 104°C.

Examples 13 and 14

Following the same procedure as described in Example 12 above, the following were prepared: Example 13, δ -cyano-8-amino-3,4-dihydroxy-5-methoxycinnamal malononitrile, m.p.
35

225°C; and Example 14, γ -cyano- β -amino-3,4-dihydroxy-5-bromocinnamal malononitrile, m.p. 241°C.

Example 15

4-Formylbenzylidene malononitrile

5 To 3.1 g (15 mmol) terephthalaldehyde monoethylacetal and 1.1 g (16.5 mmol) malononitrile in 30 ml ethanol were added 6 drops piperidine. After 1 hour of reflux, 50 ml of 1 N HCl was added and the reaction mixture was heated at 70°C for 20 minutes, cooled and filtered to give 2.3 g (85% yield)
10 of a yellow solid, m.p. 142°C.

Example 16

3,4-Dihydroxybenzylidenecyanoacethydrazide

To 0.69 g (5 mmol) 3,4-dihydroxybenzaldehyde and 0.55 g (5.5 mmol) cyanoacethydrazide in 40 ml ethanol was added 2
15 drops piperidine. After 2½ hours of reflux, the reaction mixture was cooled, filtered and washed with cold ethanol to give 0.55 g (50% yield) of product, an orange solid, m.p. 220°C (decomposition).

Example 17

4-Methoxycinnamal malononitrile

(a) To 2 g (12.6 mmol) 4-methoxycinnamalonitrile in 10:10:20 ml H₂O:HOAc:pyridine was added 5 g (47 mmol) NaH₂PO₂ (hypophosphite) followed by 2 g Ra-Ni. After stirring 3 hours at 50°C, the reaction mixture was filtered, extracted
25 with CH₂Cl₂, washed with dilute HCl, washed with H₂O and evaporated to give 0.7 g (35% yield) 4-methoxycinnamaldehyde, a yellow solid, m.p. 47°C.

(b) To 0.52 g (3.2 mmol) of the aldehyde from step (a) and 0.26 g (4 mmol) malononitrile in 30 ml ethanol was added
30 2 drops piperidine and the reaction mixture was refluxed for 40 minutes, cooled, filtered and washed with ethanol to yield 0.61 g (90% yield) of an orange solid, m.p. 160°C.

Example 183,4-Dihydroxybenzylidenecyanoacetylurea

To 0.69 g (5 mmol) 3,4-dihydroxybenzaldehyde and 0.6 g (5.3 mmol) cyanoacetylurea in 40 ml ethanol was added 2 drops
5 piperidine. The reaction mixture was refluxed for 2 hours, cooled, filtered and washed with cold ethanol to yield 0.75 g (60% yield) of a yellow-orange solid, m.p. 235°C (decomposition).

Example 1910 5-Methoxy-1-(cyano tert-butylcarboxymethylene)indane

To 1 g (6 mmol) 5-methoxyindanone and 1.6 g (11 mmol) tert-butylcyanoacetic acid ester in 30 ml ethanol was added 0.3 g β -alanine and the reaction mixture was refluxed for 30 minutes, evaporated and chromatographed on silica gel.

15 Recrystallization from ethanol gave 0.45 g (25% yield) of a white solid, m.p. 132°C.

Examples 20 and 21

Following the same procedure as set forth for Example 19 above, the following were prepared: Example 20, ethyl 8-(3,5-
20 di-tert butylphenyl)propenoic acid, m.p. 62-65°C; and ethyl α -cyano-8-(3,5-di-tert butylphenyl)propenoic acid, m.p. 94-96°C.

Example 225,6-Dihydroxy-1,1-dicyanomethyleneindane

25 (a) Caffeic acid, 1.7 g (9 mmol) in 60 ml ethanol with 0.2 g 5% Pd/C was hydrogenated in a Parr apparatus for 20 hours. Filtering and solvent evaporation gave 1.37 g (81% yield) of 3,4-dihydroxyphenylpropionic acid, a white solid, m.p. 125°C.

30 (b) The propionic acid from step (a), 2 g, in 20 ml neat liquified HF was stirred for 24 hours in a Kelf system at room temperature. The HF was vaporated and a gray solid was extracted with ethyl acetate and chromatographed on silica gel to give 0.26 g (14% yield) of 5,6-dihydroxy-1-
35 indanone, a light brown-white solid.

- (c) To the indanone from step (b), 0.25 g (1.5 mmol), in 30 ml ethanol and 0.2 g (3 mmol) malononitrile was added 80 mg β -alanine and the reaction mixture was refluxed for 20 hours and evaporated to yield a yellow-brown solid, m.p. 185°C.

Example 23

5-Hydroxy-1,1-dicyanomethylene-1,2,3,4-tetrahydronaphthalene

- To 0.5 g (3 mmol) 5-hydroxytetralone and 0.4 g (6 mmol) malononitrile in 40 ml ethanol was added 4 drops piperidine. The reaction mixture was refluxed for 14 hours, H_2O/HCl was added, and the product was extracted with CH_2Cl_2 . Chromatography on silica gel gave 30 mg of product, a yellow solid, m.p. 140°C.

Example 24

α -(3,4-Dihydroxystyryl)-4-toluylacetonitrile

- To 0.7 g (5 mmol) 3,4-dihydroxybenzaldehyde and 0.8 g (5 mmol) toluylacetonitrile in 40 ml ethanol were added 4 drops piperidine and the reaction mixture was refluxed for 1½ hours and cooled. 100 ml H_2O and 5 ml HCl were added and the product was extracted with CH_2Cl_2 and triturated with CCl_4 to give 0.4 g (28% yield) of product, a yellow solid, m.p. 172°C.

Example 25

N-Phenyl- α -cyano-3,4-dihydroxycinnamamide

- Following the procedure as set forth in Example 6 above, N-phenylcyanoacetamide was condensed with 3,4-dihydroxybenzaldehyde to yield the title compound, m.p. 258°C.

Example 26

α -Cyano-8-(3,5-di-tert butylphenyl)propenethioamide

- Following the procedure as set forth in Example 11 above, cyanothioacetamide was condensed with 3,5-di-tert butyl-benzaldehyde to yield the title compound, m.p. 172-174°C.

All compounds gave correct analytical and spectroscopic data.

EGFR Inhibition Tests

Tests on extracted EGF Receptors:

- 5 EGF receptors were prepared from A431 cells (obtained from the ATCC) and PTK activity of these receptors was assayed as described by S. Braun, W.E. Raymond and E. Racker, J. Biol. Chem. 259, 2051-2054 (1984). Various compounds useful in practicing the invention were tested for their
- 10 inhibitory capacity on the EGF-receptor kinase activity, using the assay described above. Figure 1 demonstrates characteristic results using 10 compounds. The assay conditions were as described above using 0.125 mg of copoly Glu⁶Ala³Tyr¹. Dissociation constants were calculated from the
- 15 inhibition curves and are indicated for each formula in Figure 1.

Table 1 below summarizes the results of the above-described assay, and shows K_{inh}, the dissociation constant of the PTK-inhibitor complex, as expressed in μ M units. The

20 different K_{inh} values were determined by the analysis according to Dixon.

TABLE 1

	Example	K _{inh} , μ mol
	1	11 \pm 0.1
25	2	2.2 \pm 0.3
	5	4.5
	6	3.5 \pm 0.6
	7	*
	8	*
30	9	*
	10	23.6
	11	0.85
	13	*
	14	*
35	15	20

* non-competitive inhibitor

The results of this assay indicate that compounds of the present invention competitively inhibit EGF receptor kinase

at the substrate site of the tyrosine kinase domain of EGF receptors.

Tests on cells in tissue culture:

a) A431 cells and KB cells express EGF receptors on their cell surface and their growth rate depends on the presence of growth factors in the medium. These cells were seeded and grown as described in O. Kashles and A. Levitzki, Biochem. Pharmacol., 35, 1531-1536 (1987). The compounds, the formulae of which are given in Figure 2, were added to the medium at a cell concentration of about 2×10^5 cells/well. The inhibitor was added to the medium 1 hour after seeding. The medium volume in each well was 1 ml and the concentration of inhibitor therein 20 μ M. Every 24 hours cells were counted and fresh medium with inhibitor was applied to the remaining wells. The growth curves were determined in 24-well Costar dishes.

b) Some of the compounds according to the present invention are exclusive inhibitors to EGF-dependent growth of cells and others are preferential inhibitors to such growth. Examples of the former are depicted in Figure 4b and of the latter in Figure 4a. In the experiment depicted in these Figures, 25,000 cells per well were placed in a 24 well plate (Costar) supplied with Dulbecco medium containing 10% foetal calf serum, with 10 ng/ml EGF (filled symbols \bullet , \blacksquare and \blacktriangle) or with no added EGF (open symbols \circ , \square and \triangle). EGF receptor kinase inhibitors at various concentrations were added to the cells two hours after plating. The medium containing the inhibitors was replaced with fresh inhibitor-containing medium every other day. On the fifth day, the number of cells in the presence of EGF and in the absence of EGF was determined. In Figures 4a and 4b "100%" refers to the number of cells in the absence of inhibitor for each mod of cell growth (without EGF: $100,000 \pm 10,000$ cells; with EGF: $260,000 \pm 30,000$ cells for seven experiments). The filled symbols in Figures 4a and 4b refer to inhibition of EGF-stimulated growth, whereas the open symbols depict inhibition

of EGF-independent growth. Each experimental point represents the average of triplicate determination where the variance was less than 5 percent. The compound numbers refer to compounds in Figure 1.

5 Compounds of this invention are subjected to various biological tests, the results of which correlate to useful cellular antiproliferative activity. These tests are useful in determining EGF receptor kinase and insulin receptor kinase inhibition activities of the compounds disclosed
10 herein.

EGF-Receptor Purification

EGF-receptor purification is based on the procedure of Yarden and Schlessinger. A431 cells are grown in 80 cm² bottles to confluency (2 x 10⁷ cells per bottle). The cells
15 are washed twice with PBS and harvested with PBS containing 1.0 mmol EDTA (1 hour at 37°C), and centrifuged at 600g for 10 minutes. The cells are solubilized in 1 ml per 2 x 10⁷ cells of cold solubilization buffer (50 mmol Hepes buffer, pH 7.6, 1% Triton X-100, 150 mmol NaCl, 5 mmol EGTA, 1 mmol
20 PMSF, 50 µg/ml aprotinin, 25 mmol benzamidine, 5 µg/ml leupeptin, and 10 µg/ml soybean trypsin inhibitor) for 20 minutes at 4°C. After centrifugation at 100000g for 30 minutes, the supernatant is loaded onto a WGA-agarose column (100 µl of packed resin per 2 x 10⁷ cells) and shaken for 2
25 hours at 4°C. The unabsorbed material is removed and the resin washed twice with HTN buffer (50 mmol Hepes, pH 7.6, 0.1% Triton X-100, 150 mmol NaCl), twice with HTN buffer containing 1 M NaCl, and twice with HTNG buffer (50 mmol Hepes, pH 7.6, 0.1% Triton X-100, 150 mmol NaCl, and 10%
30 glycerol). The EGF receptor is eluted batchwise with HTNG buffer containing 0.5 M N-acetyl-D-glucosamine (200 µl per 2 x 10⁷ cells). The eluted material is stored in aliquots at -70°C and diluted before use with TMTNG buffer (50 mmol Tris-Mes buffer, pH 7.6, 0.1% Triton X-100, 150 mmol NaCl, 10%
35 glycerol).

EGFR Kinase Catalyzed Phosphorylation
of Poly(GAT) and its Inhibition

WGA-purified EGFR (0.25 µg/assay) is preactivated with EGF (0.85 µM) in 50 mmol Tris-Mes buffer, pH 7.6 for 20 minutes at 4°C. The assay is initiated by addition of a mixture which contains Mg(Ac)₂ (60 mmol), [γ -³²P]ATP (125 µM, 2-5 µCi/assay), poly(GAT) (0.0625 mg/ml, 0.125 mg/ml, 0.25 mg/ml), and six concentrations of inhibitor in duplicates. The temperature of the assay is 22°C and the production of phosphorylated copolymer is found to be linear up to 20 minutes. The PTK inhibitors tested are solubilized in water or a mixture of ethanol and water such that the final concentration of ethanol does not exceed 4% in the assay. Up to 4% ethanol in the assay has no effect on the EGFR kinase activity. The concentration of EGF in the assay is 300 nM in a final volume of 40 µl. After 5, 10 or 20 minutes, aliquots of 25 µl are applied onto Whatman 3-mm paper cuttings, which are then soaked in cold 10% TCA containing 0.01 M sodium pyrophosphate. After being washed overnight at 4°C, the paper cuttings are dried and counted, measuring ³²P Cerenkov radiation. Concentration dependence on poly(GAT) was Michaelian with a $K_m = 0.076 \pm 0.007$ mg/ml or 0.069 ± 0.007 mmol if calculated per Glu₆Ala₃Tyr(GAT) unit. The EGF response for the poly(GAT) phosphorylation is graphed. The K_m for ATP in the assay was found to 2.9 µM.

Time Dependence of EGF-Receptor Autophosphorylation

WGA-purified EGF receptor from A431 cells (0.5 µg assay) is activated with EGF (800 nM) for 20 minutes at 4°C. The reaction is initiated by the addition of Mg(Ac)₂ (60 mmol), Tris-Mes buffer, pH 7.6 (50 mmol), and [³²P]ATP (20 µM, 5 µCi/assay). The reaction is conducted at either 4 or 15°C and terminated by addition of sodium dodecyl sulfate (SDS) sample buffer (10% glycerol, 50 mmol Tris, pH 6.8, 5% 8-mercapto-ethanol, and 3% (SDS). The samples are run on a 8% SDS polyacrylamide gel (SDS-PAGE) (prepared from 30% acrylamide and 0.8% bis-(acrylamide) and contained 0.375 M

Tris, pH 8.8, 0.1% SDS, 0.05% TEMED, and 0.46% ammonium persulfate). The gel is dried and autoradiography performed with Agfa Curix RP2 X-ray film. The relevant radioactive bands are cut and counted in the Cerenkov mode. The fast
5 phase of autophosphorylation continues for another 10 minutes. The extent of phosphorylation completed in the first 10-s at 15°C comprises 1/3 of the total autophosphorylation signal and probably reflects the phosphorylation of the first site on the receptor. The 10-s
10 interval is therefore chosen for use in subsequent autophosphorylation experiments.

ATP and EGF Dependence of Autophosphorylation

WGA-purified EGF receptor from A431 cells (0.5 µg/assay) is activated with EGF (0.85 µM) for 20 minutes at 4°C. The
15 assay is performed at 15°C and initiated by addition of Mg(Ac)₂ (60 mmol), Tris-Mes buffer, pH 7.6 (50 mmol), [³²P]ATP (carrier free, 5 µCi/assay), and increasing concentrations of nonradioactive ATP. The assay is terminated after 10-s by addition of SDS sample buffer. The samples are run on a 6%
20 SDS polyacrylamide gel. The gel is dried and autoradiographed as described above. The relevant radioactive bands are cut and counted in the Cerenkov mode. the K_m for ATP determined in this fashion is found to be 7.2 µM. With use of the 10-s assay protocol, the EGF
25 concentration dependence of EGFRK autophosphorylation is determined.

Inhibition of Copoly(Glu,Tyr) Phosphorylation by Insulin-Receptor Kinase (InsRK)

Rat liver membranes are prepared from the livers of 6-
30 week-old rats as described by Cuatrecasas. WGA-purified insulin receptor is prepared according to Zick et al. WGA-purified rat liver InsRK (1.25 µg) is preincubated with or without 330 nM insulin in 50 mmol Tris-Mes buffer, pH 7.6, for 30 minutes at 22°C. The assay is performed at 22°C and
35 initiated by addition of a mixture which contains Mg(Ac)₂ (60

- mmol), NaVO_3 (40 μM), [γ - ^{32}P]ATP (125 μM , 3-5 $\mu\text{Ci}/\text{assay}$), and poly(GT) [poly(Glu₄Tyr)] at three concentrations: whenever an inhibitor is tested, it is added at the proper concentration. The final concentration of insulin in the assay is 125 nM.
- 5 The total volume of the assay is 40 μl . After 20 minutes, aliquots of 30 μl are applied on Whatman 3-mm paper and soaked in cold 10% TCA, containing 0.01 M sodium pyrophosphate. After being washed overnight, the papers are dried and counted, measuring Cerenkov radiation. The InsRk-
- 10 catalyzed phosphorylation of poly(GT) obeys Michaelis-Menten kinetics.

Inhibition of EGFR Autophosphorylation

- A431 cells were grown to confluence on human fibronectin coated tissue culture dishes. After washing 2 times with
- 15 ice-cold PBS, cells were lysed by the addition of 500 $\mu\text{l}/\text{dish}$ of lysis buffer (50 mmol Hepes, pH 7.5, 150 mmol NaCl, 1.5 mmol MgCl_2 , 1 mmol EGTA, 10% glycerol, 1% triton X-100, 1 mmol PMSF, 1 mg/ml aprotinin, 1 mg/ml leupeptin) and incubating 5 minutes at 4°C. After EGF stimulation (500 $\mu\text{g}/\text{ml}$ 10 minutes
- 20 at 37°C) immunoprecipitation was performed with anti EGF-R (Ab 108) and the autophosphorylation reaction (50 μl aliquots, 3 μCi [γ - ^{32}P]ATP) sample was carried out in the presence of 2 or 10 μM of compound, for 2 minutes at 4 °C. The reaction was stopped by adding hot electrophoresis sampl
- 25 buffer. SDS-PAGE analysis (7.5% gel) was followed by autoradiography and the reaction was quantitated by densitometry scanning of the x-ray films.

Inhibition of Cell Proliferation as Measured by Inhibition of DNA Synthesis

- 30 Cells were seeded at 1×10^5 cells per well in 24-well Costar dishes pre-coated with human fibronectin (by incubating for 30 minutes at room temperature with 10 $\mu\text{g}/0.5$ ml/well). The cells were grown to confluence for 2 days. The medium was changed to DMEM containing 0.5 calf serum for
- 35 36-48 hours and the cells were then incubated with EGF

(Toyobo, New York, NY) (20 ng/ml) or serum (10% calf serum) and different concentrations of the inhibitory compounds. [³H]thymidine, (NEN, Boston, MA) was added 16-24 hours later at 0.5μCi/ml for 2 hours. TCA precipitable material was

5 quantitated by scintillation counting (C). Results of this assay are summarized in Table II below. "IC₅₀," as used below, refers to the concentration of inhibitor (μM) at which [³H]thymidine incorporation is halved, compared with media containing no inhibitor. As FCS contains a broad range of

10 growth factors, the IC₅₀ values for EGF should be lower than for FCS, indicating that the compounds do not act as general inhibitors.

TABLE II

	<u>Example</u>	<u>IC₅₀ - EGF</u>	<u>IC₅₀ - FCS</u>
15	3	0.1	0.2
	21	20	120
	25	30	100
	26	20	100

These results indicate that the compounds of the

20 invention do not inhibit a broad range of growth factor receptors.

Cell Culture

Cells termed HER 14 and K721A (=DK) were prepared by transfecting NIH3T3 cells (clone 2.2) (From C. Fryling, NCI,

25 NIH), which lack endogenous EGF-receptors, with cDNA constructs of wild-type EGF-receptor or mutant EGF-receptor lacking tyrosine kinase activity (in which Lys 721 at the ATP-binding site was replaced by an Ala residue, respectively). All cells were grown in DMEM with 10% calf

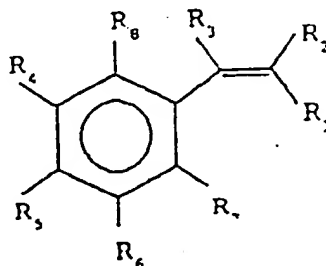
30 serum (Hyclone, Logan, Utah).

The results obtained by the above experimental methods evidence the useful protein tyrosine kinase inhibition properties of the compounds within the scope of the present invention.

We claim:

1. A method for inhibiting cell proliferation in a patient comprising administering to said patient an effective amount of a composition comprising, in admixture with a pharmaceutically acceptable carrier, a compound, or a pharmaceutically acceptable salt thereof, selected from the group consisting of

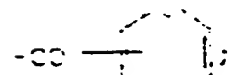
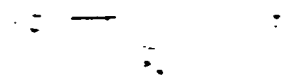
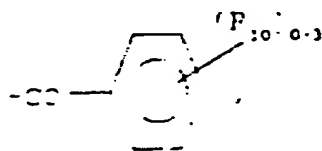
(A) a substituted styrene compound having the formula



wherein:

one of R_1 and R_2 is alkyl, -H, -CN or -OH;

the other of R_1 and R_2 is alkyl, -H, -CN, -OH, -CHO, -CONHR₉, -CONHCH₂CN, -CH=C(CN)₂, -NHCHO,



wherein R_1 and R_2 are not both alkyl, -H, -CN or -OH;

R_3 is alkyl, -H, -CN, -OH, -COOR, -CONRR, -CSNRR, -CH₂CN or -CH=C(CN)CONH₂;

R_4 is alkyl or -H;

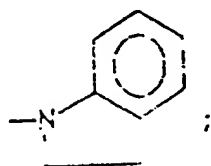
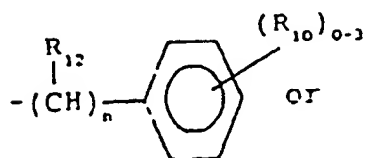
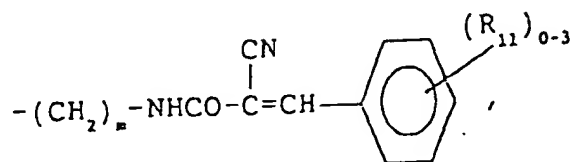
R_4 , R_5 , R_6 , R_7 and R_8 are each independently -CN, -OR, -COOH,

-NHCOCH₃, -SR, -CH=CHCOOH, -NHCO(CH₂)₂COOH or morpholino;

R_3 and R_7 together may be -CH₂CH₂-, -CH₂CH₂CH₂- or, starting from

R_3 , -CONH-;

R_9 is -NH₂, -CONH₂,



R_{10} is alkyl, halo, -OR, -COOR or -NO₂;

R_{11} is alkyl, -CN, halo, -OR, -CHO, -COOH, -NRR, -NO₂, -NHCOCH₃, -SR, -CH=CHCOOH, -NHCO(CH₂)₂COOH or morpholino;

R_{12} is alkyl, -H, halo, -OR or -COOR;

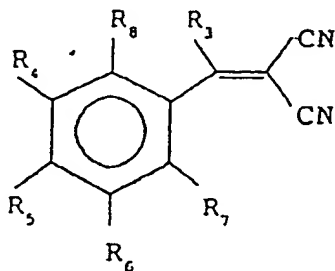
n is 0 to about 6; and

m is 2 to about 10;

provided that when one of R_1 and R_2 is -CH=C(CN)₂, at least one of R_3 , R_4 , R_5 , R_6 , R_7 and R_8 is not -H; and

in the case that R_3 and R_7 together are $-\text{CH}_2\text{CH}_2-$, $-\text{CH}_2\text{CH}_2\text{CH}_2-$ or, starting from R_3 , $-\text{CONH}$, R_1 and R_2 may also independently be $-\text{COOR}$, $-\text{CONRR}$ or $-\text{CSNRR}$; and

(B) a bicyclic benzylidene malononitrile compound having the for: 1a



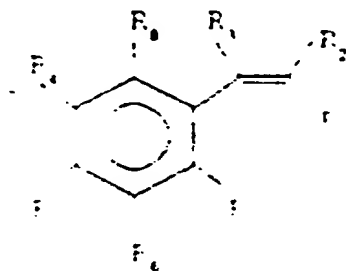
wherein:

R_3 and R_7 together are $-\text{CH}_2\text{CH}_2-$, $-\text{CH}_2\text{CH}_2\text{CH}_2-$ or, starting from R_3 , $-\text{CONH}-$;

R_4 , R_5 , R_6 and R_8 are each independently alkyl, $-\text{H}$, $-\text{CN}$, halo, $-\text{OR}$, $-\text{CHO}$, $-\text{COOH}$, $-\text{NRR}$, $-\text{NO}_2$, $-\text{NHCOCH}_3$, $-\text{SR}$, $-\text{CH}=\text{CHCOOH}$, $-\text{NHCO}(\text{CH}_2)_2\text{COOH}$ or morpholino; and

R is alkyl or $-\text{H}$.

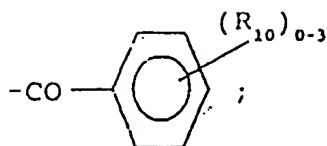
2. A method according to Claim 1 wherein said compound has the formula



wherein:

R_1 is $-\text{CN}$;

R_2 is $-\text{H}$, $-\text{CN}$, $-\text{CONHR}$, or



wherein R_1 and R_2 are not both $-\text{CN}$;

R is alkyl or $-\text{H}$;

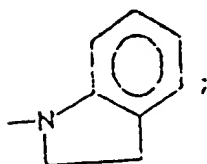
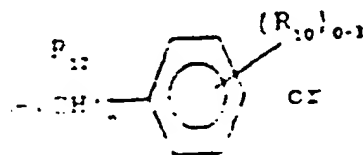
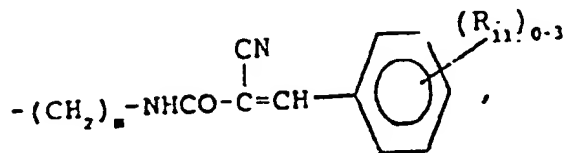
R_3 is $-\text{H}$;

R_4 , R_5 , R_6 , R_7 and R_8 are each independently alkyl, $-\text{H}$, halo or $-\text{OR}$;

R_3 and R_7 together may be $-\text{CH}_2\text{CH}_2-$, $-\text{CH}_2\text{CH}_2\text{CH}_2-$ or, starting from

R_3 , $-\text{CONH}-$;

R_9 is $-\text{NH}_2$, $-\text{CONH}_2$,



R_{10} is alkyl, halo, $-\text{OR}$, $-\text{COOR}$ or $-\text{NO}_2$;

R_{11} is alkyl, halo or -OR;

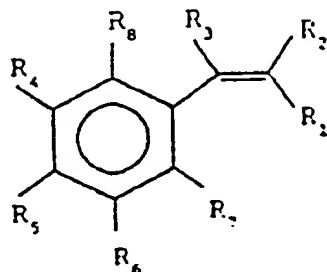
R_{12} is alkyl or -H;

n is 0 to about 4;

m is 2 to about 6; and

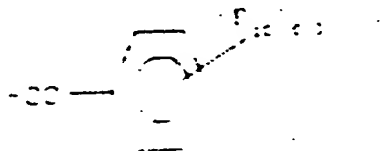
in the case that R_3 and R_7 together are $-\text{CH}_2\text{CH}_2-$, $-\text{CH}_2\text{CH}_2\text{CH}_2-$ or, starting from R_3 , $-\text{CONH}$, R_1 and R_2 may independently be $-\text{COOR}$, $-\text{CONRR}$ or $-\text{CSNRR}$.

3. A method according to Claim 2 wherein said compound has the formula



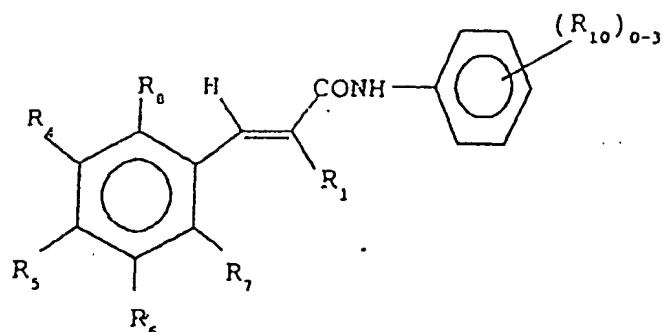
wherein:

one of R_1 and R_2 is $-\text{CN}$, with the proviso that R_1 and R_2 are not both $-\text{CN}$; and
the other of R_1 and R_2 is $-\text{CONHR}$, or

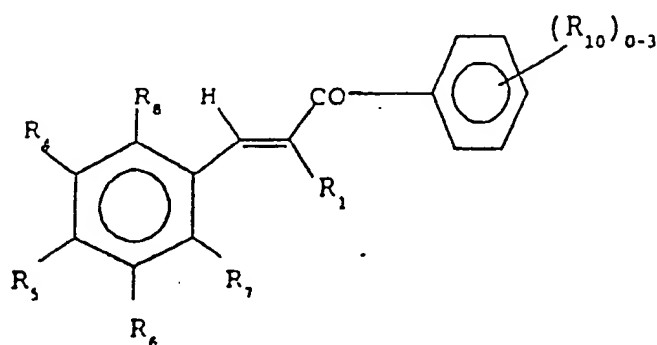


4. A method according to Claim 2 wherein R_1 is $-\text{CN}$.

5. A method according to Claim 3 wherein said compound has the formula



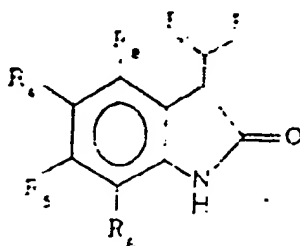
6. A method according to Claim 3 wherein said compound has the formula



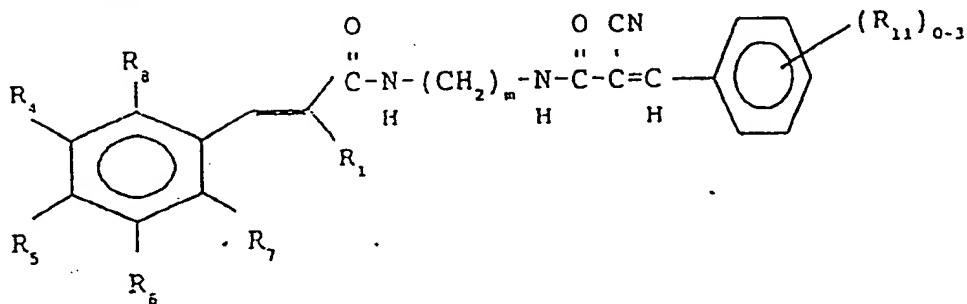
7. A method according to claim 5 wherein R_1 is $-CN$; R_4 and R_5 are each independently $-OH$; R_6 , R_7 , and R_8 are each independently $-H$; and there are no R_{10} substituents.

8. A method according to Claim 6 wherein R_1 is $-CN$; R_4 and R_5 are each independently $-OH$; R_6 , R_7 , and R_8 are each independently $-H$; and there are no R_{10} substituents.

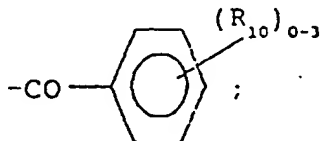
9. A method according to Claim 1 wherein said compound has the formula



10. A method according to Claim 2 wherein said compound has the formula



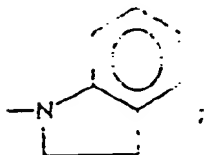
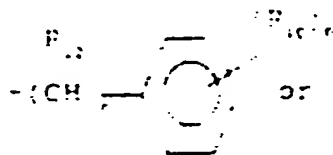
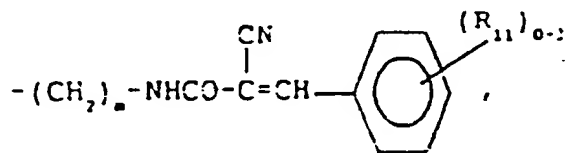
11. A method according to Claim 2 wherein R_2 is $-H$, $-CONHR_9$ or



R is alkyl or $-H$;

R_4 , R_5 , R_6 , R_7 and R_8 are each independently lower alkyl, $-H$, $-OH$, lower alkoxy or halo;

R_9 is $-NH_2$, $-CONH_2$,

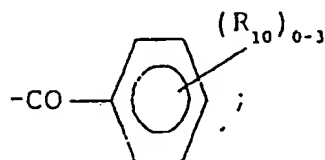


R_{10} is lower alkyl or $-COOR$;

R_{11} is lower alkyl, $-OH$, lower alkoxy or halo; and

m is 2 to about 10.

12. a method according to Claim 2 wherein
 R_1 is -CN or



R is alkyl or -H;

R_4 , R_5 , R_6 , R_7 and R_8 are each independently lower alkyl, -H, -OH, lower alkoxy or halo; and

R_{10} is lower alkyl or -COOR.

13. A method according to Claim 9 wherein
 R_1 is -CN;

R_2 is -H, -CN, -CONHR, or

wherein R_1 and R_2 are not both -CN;

R is alkyl or -H;

R_4 , R_5 , R_6 and R_8 are each independently lower alkyl, -H, -OH, lower alkoxy or halo; and

R_{10} is lower alkyl or -COOR.

14. A method according to Claim 10 wherein
 R_1 is -CN;

R is alkyl or -H;

R_4 , R_5 , R_6 and R_8 are each independently lower alkyl, -H, -OH, lower alkoxy or halo;

R_{11} is lower alkyl, -OH, lower alkoxy or halo; and

m is 2 to about 6.

15. A method for inhibiting cell proliferation of a patient suffering from such disorder comprising administering to said patient an effective amount of a composition comprising, in admixture with a pharmaceutically acceptable salt thereof, selected from the group consisting of ethyl β -(3,5-di-tert butylphenyl)propenoic acid and ethyl α -cyano- β -(3,5-di-tert butylphenyl)propenoic acid.

16. A method according to Claim 1 wherein said compound is ethyl β -(3,5-di-tert butylphenyl)propenoic acid.

17. A method according to Claim 1 wherein said compound is ethyl α -cyano- β -(3,5-di-tert butylphenyl)propenoic acid.

18. A method according to Claim 1 wherein said compound is N-phenyl- α -cyano-3,4-dihydroxycinnamamide.

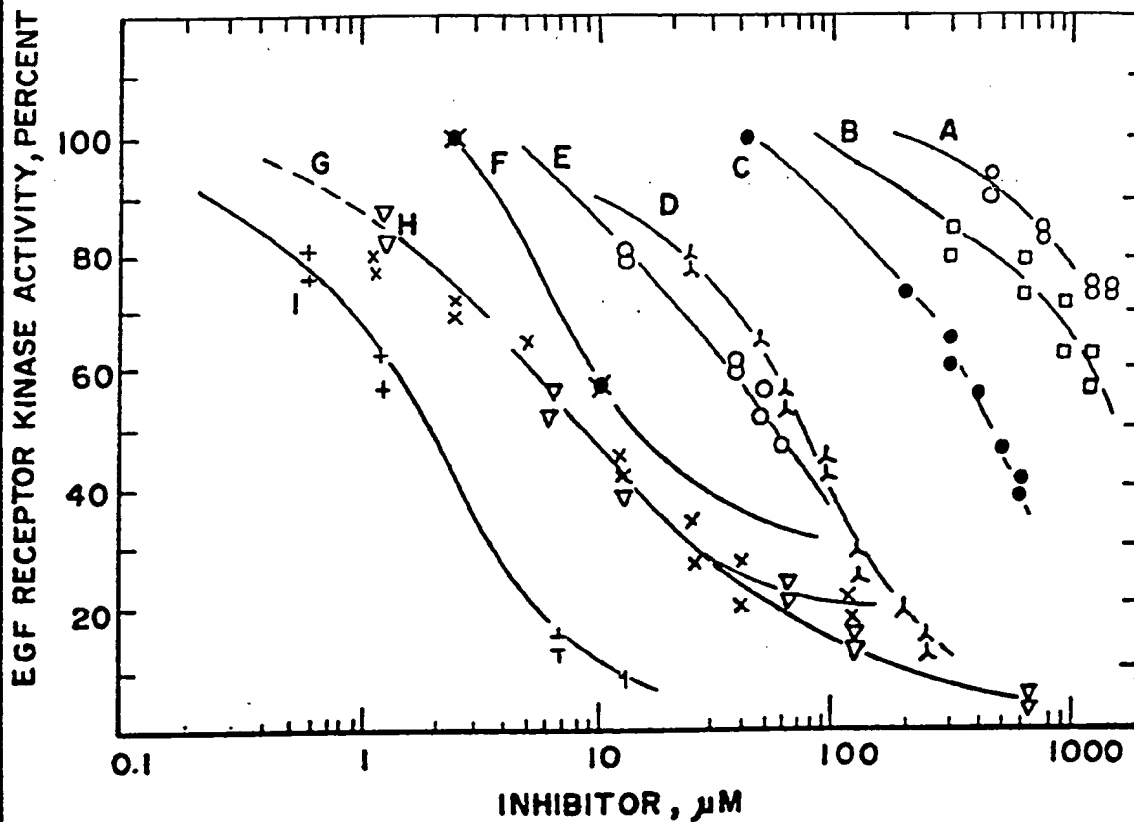
19. A method according to Claim 1 wherein said compound is α -cyano- β -(3,5-di-tert butylphenyl)propenethioamide.

20. A method for the treatment of psoriasis in a patient suffering from such disorder comprising administering to said patient an effective amount of the composition of Claim 1.

21. A method for the treatment of atherosclerosis in a patient suffering from such disorder comprising administering to said patient an effective amount of the composition of Claim 1.

1 / 4

FIG. 1



SUBSTITUENTS

COMPOUND	R ₃	R ₄	R ₅	R ₆	R ₇	R ₂	R ₁	K _{inh} μM
A	II	H	OH	H	H	CO ₂ H	H	1000
B	II	H	OH	H	H	CO ₂ H	CO ₂ H	500
C	II	OH	OH	H	H	CO ₂ H	H	150
D	II	OH	H	H	OH	CN	CO ₂ H	24
E	II	H	OH	OH	H	CO ₂ H	CN	18
F	II	H	OH	OH	H	CN	CN	11
G	II	OHC ₃	OH	OH	H	CN	CN	2
H	II	H	OH	OH	H	CONH ₂	CN	2.3
I	II	H	OH	OH	H	CSNH ₂	CN	0.85

SUBSTITUTE SHEET

2 / 4

FIG. 2a

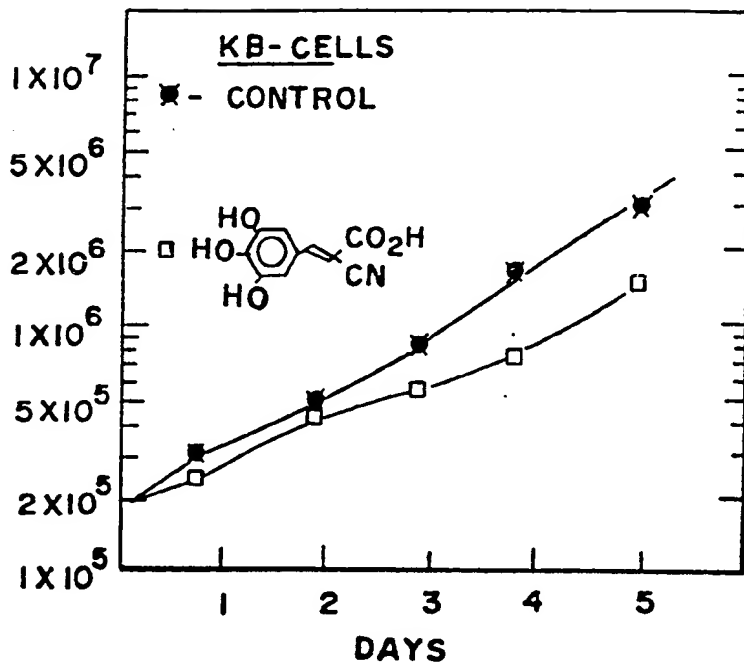
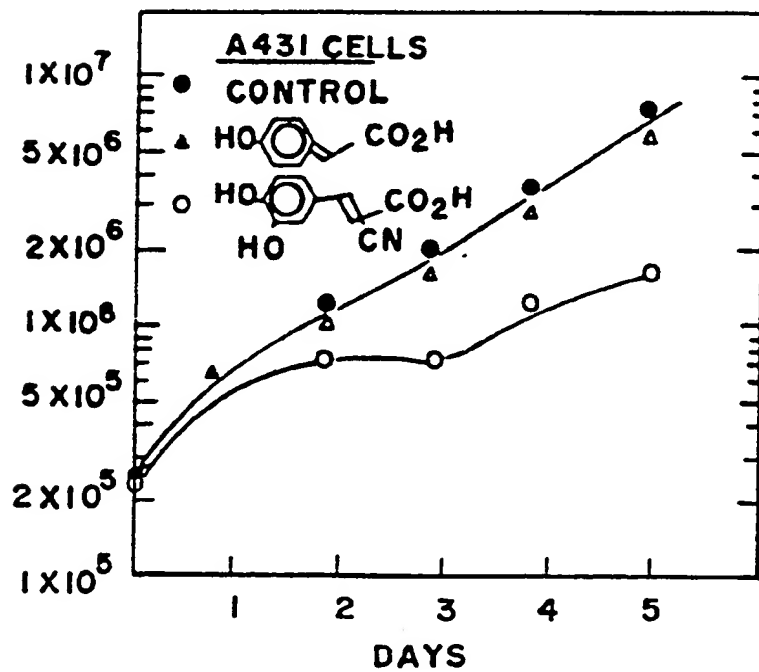


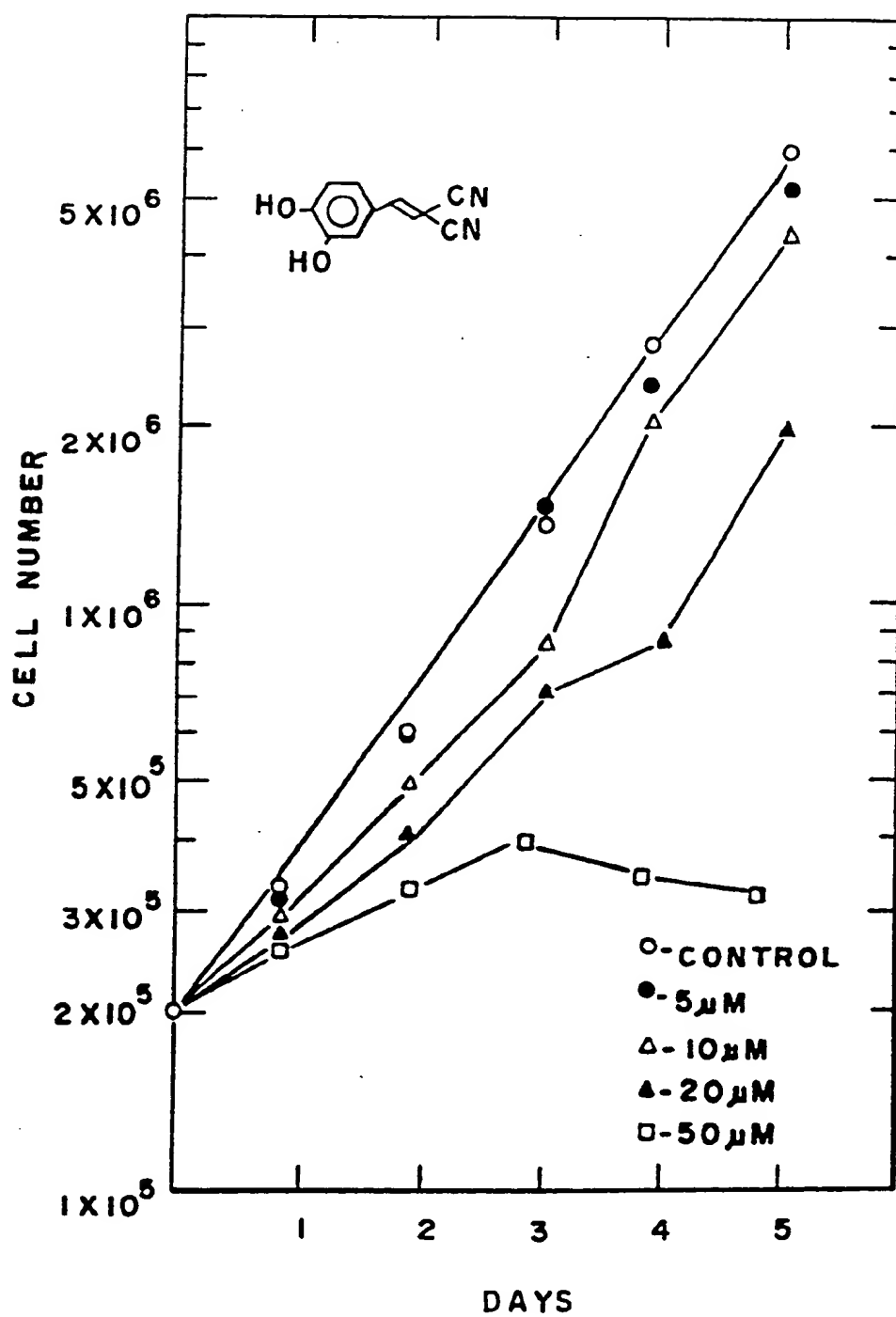
FIG. 2b



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3 / 4

FIG. 3



4 / 4

FIG. 4a

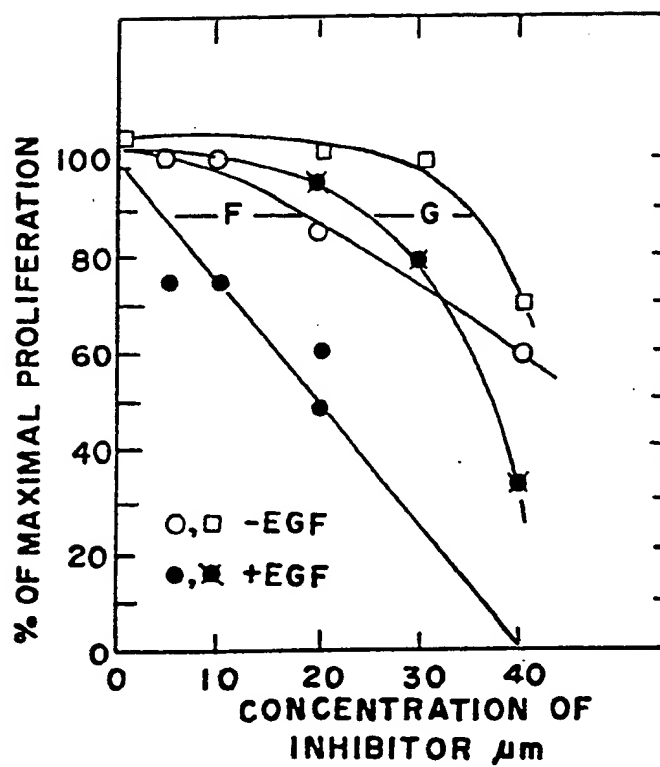
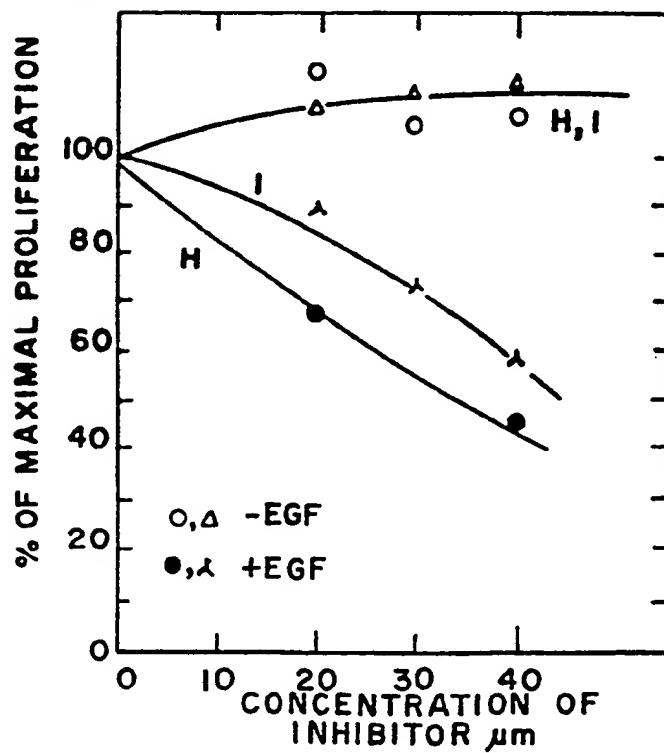
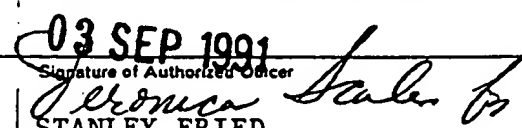


FIG. 4b



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/02931

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): A61K 31/415 U.S. CL: 514-388		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
U.S. CL.	514-388	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
CAS ON LINE		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
E	Chemical Abstracts, volume 110 507726B (1909), YAISH ET AL.	1-21
E	Chemical Abstracts, volume 111, 187005F (1989), LYALL ET AL.	1-21
<div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p>¹⁴ Special categories of cited documents ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another claim or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATE		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
29 JULY 1991		03 SEP 1991
International Searching Authority		Signature of Authorized Officer
ISA/US		 STANLEY FRIED